Stavrianopoulos et al., Serial No. 08/486,070 (Filed June 7, 1995)
Exhibit 2 [Fifth Supplemental IDS -- February 6, 2006]

EXHIBIT 2

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I, Jessica Majestic, hereby certify that the following is, to the best of my knowledge and belief, a true and accurate translation of the International Patent Classification C07H 19/20; G01N 53/50 C12Q 1/00 with International Publication No.: WO 83/02276 and International Publication Date: July 7, 1983 (07.07.83) from French into English.

Signature

Sworn to before me this
3rd day of February, 2006

Signature, Notary Public

HEATHER BOSLEY
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(54) Title: MODIFIED ADENOSINE 5'-TRIPHOSPHORIC ACID AND METHOD FOR DOSING BIOLOGICAL SUBSTANCES CAPABLE OF FIXING DEGRADATION PRODUCTS OF ADENOSINE 5'-TRIPHOSPHORIC ACID

(57) Abstract

Modified ATP capable of originating an ADP or AMP, which is also modified and may be incorporated in biological substances, and allowing the detection and optionally, the dosing of the latter. Such modified ATP is characterized by the covalent fixation, on the adenine cycle of the ATP, of a chemical group which may be coupled directly or indirectly with a related molecule, preferably marked by an enzyme, thereby allowing the recognition of said ATP, ADP or AMP.

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Modified adenosine 5'- triphosphoric acid and method for dosing biological substances capable of fixing degradation products of adenosine 5'-triphosphoric acid.

The invention is related to a derivative of the adenosine 5'- triphosphoric acid (in other words, a derivative of ATP) and to a method for dosing biological substances capable of fixing degradation products of AT, in the presence of the appropriate enzyme; in other words, the adenosine 5'-monophosphate acid (AMP) or the adenosine 5'-diphosphate acid (ADP).

It is known that a category of biological substances, especially some antibiotics, and particularly those belonging to the aminoglycosides group are capable of fixing ADP or AMP in the presence of specific enzymes; these latter molecules are capable of being provided to this antibiotic by ATP. This fixation is described by the following chemical equation, where R-OH represents the antibiotic:

$$R\text{-}OH + ATP \xrightarrow{\text{specific enzyme}} R\text{-}O\text{-}AMP + PPi$$

The abbreviations PPi (pyrophosphate) and Pi (phosphate) are sometimes used in biology to designate the "inorganic" derivatives of phosphorus that are freed during the biological reaction of the type shown above.

This type of reaction is definitely used in the dosing of biological substances of the type shown above, but the known methods of detection become unusable when these biological substances are present only in little quantities in the samples studied.

The objective of the invention is to supply reagents that are sufficiently sensitive and permit exact utilization of this reaction to dose such biological substances, even if they do not exist in the biological samples studied in minute quantities, such as nanogram or even lesser.

According to the invention, the reagent has an ATP modification product, which can be characterized by the covalent fixation on this ATP of a chemical molecule carrying at least one free group in the above mentioned covalent fixation with a molecule or product with a specific affinity for this group and consequently allowing selective recognition of the above mentioned biological substances.

This group in particular allows a chemical refining or immunological liaison with a molecule or a product, which itself is directly detected or can be detected by coupling with another developer product. In addition, this group does not change the capacity of the chosen enzyme to ensure incorporation of the modified derivative of AMP or ADP carrying the same modification groups as the initially modified ATP in or on the substance to be dosed under normal incorporation conditions of unmodified AMP or ADP on the same substance in the presence of the same enzyme.

The invention also concerns a detection method or dosing of a substance belonging to the category of substances defined above consisting of producing a reaction in the composition assumed to contain a substance like modified ATP as per the invention in the presence of the specific corresponding enzyme, in or on the said substance, if need be, after eliminating the excess modified free ATP, to place the composition obtained under the conditions allowing the eventual coupling of the modification groups carried by the modified AMP or modified ADP incorporated in the said substance, with the molecule or the product

allowing the modification group to be recognized from the initial derivative of ATP and to detect or to dose the above mentioned, eventually present substance carrying the said products of recognition.

In the case of aminoglycoside, the dosing will start a reaction which can be shown in the following equation:

$$R\text{-}OH + ATP\text{–}M \xrightarrow{\text{specific enzyme}} R\text{-}O\text{-}ADP\text{–}M + Pi$$

Or for other substances such as the sugar – 1 – phosphate:

$$R\text{-}OH + ATP\text{–}M \xrightarrow{\text{specific enzyme}} R\text{-}O\text{-}ADP\text{–}M + Pi$$

The dosing of the R-O-AMP-M compound (or R-O-ADP-M compound), after elimination of the eventual excessive ATP-M, is of considerable sensitivity. In the case of antibiotics, this method allows the dosing of nanogram quantities of antibiotics.

Among the substances capable of being dosed by such a method other than the aminoglycoside antibiotics, we can mention the sugar–1–phosphates such as the galactose–1 phosphate (study of galactosemias) and the glucose 1-phosphate, N-acetylneuraminic acid, 5-phosphoribosyl-1-phosphoric acid...

The specific enzyme used in this transformation will in general be made of an adenylyltransferase, an acetylneuraminate citidylyltransferase, a glucose 1-phosphate uridyltransferase, ATP phosphoribosyltransferase...

The chemical group linked in a covalent manner to ATP can take many different forms, as soon as it has a group that allows coupling with refined substances allowing its detection, preferably in a directly visual form, and that it does not prevent the above mentioned enzyme from catalyzing the incorporation of the derivative of AMP or ADP corresponding to the above mentioned substance.

Among the preferred chemical groups capable

of being fixed on the bases of the aforesaid ribonucleotides, we will mention all groups capable of being specifically recognized by another molecule or by a product, itself easily detectable, preferably by a method of visualization.

This other molecule or this other product, for example in an enzyme, the presence of which can be revealed by the action that it is capable of exercising on a substrate, preferably a substrate

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giving rise to coloration or discoloration reactions or generally more modification of absorption spectrum respectively detectable by colorimetry or spectrophotometry. Naturally we can resort to molecules or products including derivative groups reacting to fluorescence, to modifications of optical density, etc.; for example products including derivative groups of aminofluoren, dansyl chloride, rhodamine, etc.

Among the allocated modification groups, we will mention chemical groups whose affinity for another type of a chemical molecule is known. Among these groups of chemical modification, we can mention biotin or avidin whose mutual affinity is known, groups derived from one of these molecules that can be used in the modification of the selected ribonucleotide and the other from a molecule that can be joined with a reagent marked with an enzyme or capable of being fixed to an enzyme, for example in the conditions described in patent no. 78 10975 of INSTITUT PASTEUR filed April 13, 1978. This reagent is made of a specific antibody, for example, guided against the modified group or in a molecule with a specific affinity for the said modified group.

Among the modified groups used for the initial ribonucleotide, they also have antigens or haptens capable of being recognized by the antibodies formed earlier against these antigens or against these haptens, particularly when these were fixed earlier on a macromolecule support such as a serum albumin or a polypeptide, for example, a polylysin. Among these antigens or haptens, we can mention biotin and avidin themselves, acetyl-aminofluoren groups, peptides, hormones or prostaglandins, especially those corresponding to antiserums or specific antibodies, lectins known for their capacity to be joined with enzymes that allow them to be detected, especially peroxidases, ß-galactosidase, etc...Such serums or antibodies are available in

commercially.

But the molecule or the product presenting the characteristics of affinity for the aforesaid modification group is eventually also used only for relaying to another molecule or to another product itself capable of being detected, especially under the above mentioned conditions; for example the product presenting the affinity characteristics for the aforesaid modification group is made of an antibody, itself unmarked but recognizable by antibodies against itself, these latter antibodies themselves being joined to the enzyme capable of acting on a specific substrate under classic conditions concerning immunoenzymatic dosage.

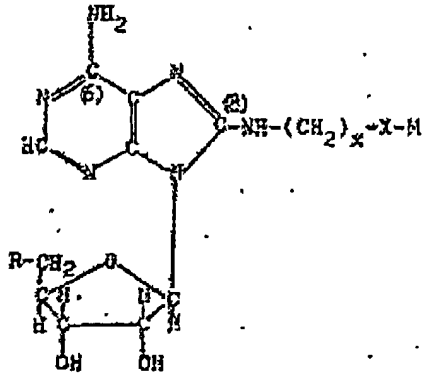
Generally, the modification group of ribonucleotide is made of any molecule or chemical product that can be fixed on the ATP and then can be detected in the indicated conditions as far as it satisfies the aforesaid conditions. The invention also includes a recognition test for compatible groups. These groups are the ones that do not prevent the fixing of the corresponding modified AMP on the aminoglycosides, especially tobramycin in the presence of adenylyl-transferase as per the aforesaid conditions; this modified AMP must naturally be supplied by the ATP already modified by the modification group studied.

The aforesaid chemical modification group is fixed in position 6, preferably 8, of the adenine group.

This fixing accordingly happens by the intermediary of an arm of the type –NH-$(CH_2)_x$-X, or –CO-$(CH_2)_x$-X, where x varies from 2 to 20, mainly from 6 to 12, and X is a group that ensures the liaison between a group M, chosen among the groups capable of a liaison reaction with a chemical or immunological agent with a selective affinity for this group. Naturally, the CH_2 groups of the aforesaid arm could be in part replaced by CO or NH groups only if

these replacement groups are not adjacent to identical groups.

For example, if we consider the case of the adenine modification group of ATP in its position 8, the modified ribonucleotide obtained perhaps can be represented by the formula (like a type arm $-NH-(CH_2)_x-X-$):



(I)

in which R is a triphosphate group, x, X and M have the aforesaid significations. Accordingly the group X is constituted by a group NH or CO.

A method to make the derivative of formula I, for example from ATP already brominated in position 8, consists of making it react under appropriate conditions with a compound formula $H_2N-(CH_2)_x-X-Y$, where Y represents a radical that will later be substituted by the aforesaid group M, especially by starting a condensation reaction with a molecule MZ, during which the condensation product of formula I is formed by freeing molecule Y-Z.

When the group X is NH, Y is accordingly hydrogen. When X is CO, Y is a hydroxyl. Z can be made of any group capable of being separated from M in the aforesaid condensation reaction, e.g., the giving chemical molecule of the researched group is fluoro-1-dinitro-2.4 benzene or a hydroxyl,

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or of hydrogen in case of a peptide. In the latter case, the fixing of this peptide on the extremity of the aforesaid arm could, when the XY group is constituted by a group NH_2 or COOH, be done by starting coupling reactions, traditional in the chemistry of proteins between carboxyl and amino groups, respectively carried by the two different peptide elements to couple, for example by condensation in the presence of a condensation agent such as dicyclohexyl-carbodiimide or after prior formation of ester activated by the carboxylic function of its two peptide elements that carry it.

The position 8 on the adenine cycle of ATP obviously does not constitute the only point on which a chain carrying a modification group such as the one defined above could be connected. As an example, we can mention the possibility of substitution, which consists of making ATP react with iodoacetic acid or an equivalent iodized organic acid, allowing the early formation of a quaternary salt, by bringing azote in position 1, a salt which will be transformed by heating in a basic environment at 35°C in a slightly pH environment, especially at pH 8 for a sufficient duration, for example 72 hours, in a substitution product of one of the hydrogens of the group NH_2 fixed on the carbon in position 6 of the adenine group (reaction of the type known as "rearrangement of Dimroth").

We then obtain (when the organic acid is made of iodoacetic acid), the compound of formula II given below:

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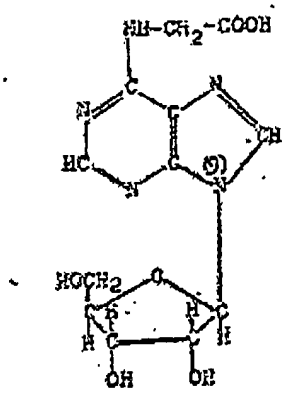

(XI)

This compound can then be transformed by reacting with the compound of the formula H_2N-$(CH_2)_X$-X-Y already defined above, as conditions permitting the chemical liaison between the carbonyl group initially contained in the compound of formula II and the imino group belonging earlier to the amino function of the compound H_2N-$(CH_2)_X$-X-Y, which can then be coupled with an MZ formula compound under conditions already defined above.

It goes without saying that all the preceding elements are simply to illustrate the particular preparation methods that allow the ATP to be fixed by a modification group selected among those that correspond to refined molecules, such as the ones defined above.

Other characteristics of the invention will be clearer during the course of the description of examples of the preferred implementation of the invention.

Preparation of 8-[-N-(dinitro-phenyl)-amino-hexyl]-aminoadenosin 5'-triphosphate

A reaction is obtained with 8-(aminohexyl)-aminoadenosin 5'-triphosphate and fluoro-1-dinitro-2.4-benzene,

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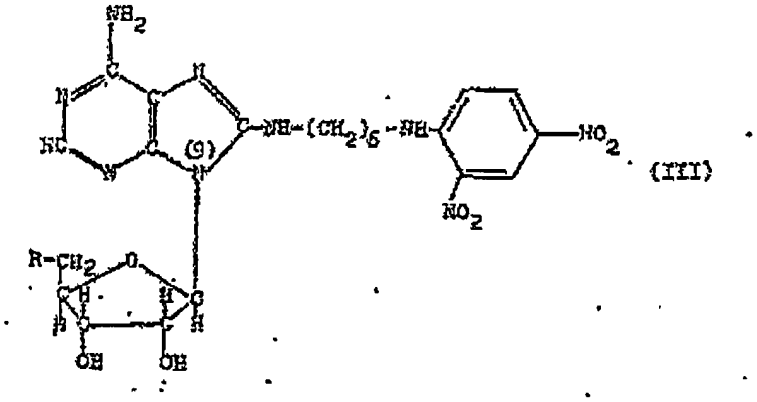
in a mix of water-ethanol 10/1 volumes %, with pH 8.8, at 40° C and in the presence of a salt, namely magnesium chloride. The product of the reaction finally obtained has the following formula:



(III)

We get the derivative of formula III, hereafter referred to as ATP-DNP, after purification, including the fixing of ribonucleotide on DEAE cellulose, elution with a gradient of LiCl 0.2 N, pH 5.5, to LiCl 0.5 N, pH 2 and filtration on a molecular screen of the type SEPHADEX G50. The reaction product is 52%. The fractions obtained are analyzed by spectrophotometer of absorption of rays of wavelengths 280 and 360 nanometers respectively. We collect those fractions whose optic density in the aforesaid two domains of wavelengths is in a ratio of (DO_{280}/DO_{360}) equals to 4. The product contained in this fraction corresponds to the one resulting from the fixing of mole 1 of DNP on mole 1 of ATP. It gives only a spot on a system of thin-section chromatography. The product of this fraction is lyophilized.

This product shows the characteristic of being recognized by the antibodies formed earlier with the dinitro-2.4 benzene, which was earlier fixed by a macromolecular support of the

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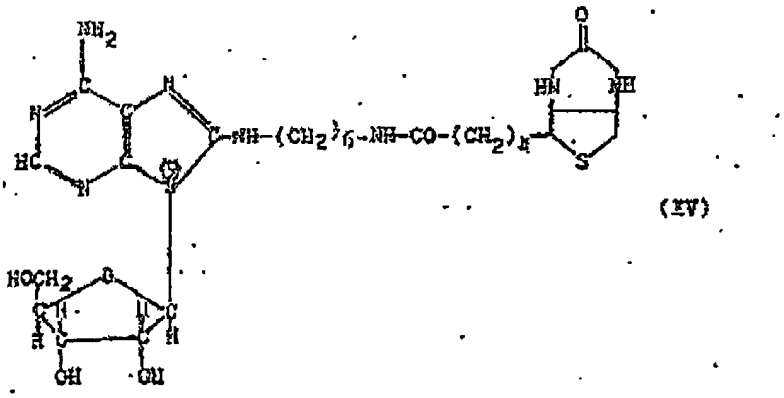
serum-albumin type. The antibodies of this kind are available commercially.

Preparation of 8-(N-biotinyl-aminohexyl)-aminoadenosine 5'-triphosphate

We condense 8-(aminohexyl)-amino-adenosine 5'-triphosphate with biotinyl-N-hydroxysuccinimide-ester, as per the conditions described by LANGER et al, and as applied to the manufacture of biotinyl-UTP from 5-(3-amino) allyluridine. We thus obtain the following compound formula:



(IV)

Tobramycin dosage

The sample assumed to contain the aforesaid antibiotic is made of a serum from a patient treated with an aminoglycoside. This serum is already de-proteinized.

This sample is placed in solution in a sealed solution with the following composition:

Tris HCl 50mM, pH 7.5

$MgCl_2$ 10mM

DTT 1 mM

To this solution we add 10 micromoles of ATP-DNP and let it incubate for 1 hour at 37° C with 10 U of adenylyl-transferase extracted from *Staphylococcus*

rivet or from *Escherichia coli* R55.

One part of the product is then taken, mainly by an EPPENDORF (GILSON) pipette that has a cation exchanger on the lower part capable of fixing the antibiotic modified by AMP-DNP; other components, including the excess ATP-DNP and the organic phosphates formed thus remain in the solution.

The cation exchanger (Amberlite CG 50, 200 – 400 mcsh, NH_4+ type) is then washed with the aforesaid sealed solution. The modified antibiotic is then eluted from the cation exchanger with the help of an ammoniac solution 1N. The solute obtained is then neutralized with HCl 1N.

Finally the dosage includes the step of putting in contact the aforesaid solution with an antibody anti-DNP coupled with peroxydase, to finally collect the complex formed between the aminoglycoside in which is incorporated the degradation product of ATP-DNP and the aforesaid antibody, and to put it in contact with a colored substrate of peroxydase. The presence of the antibiotic is then seen by the coloration of the peroxydase substrate, which even gives quantitative indications about the quantity of the antibiotic that was contained initially by the treated sample.

In the example considered, the substrate is accordingly made of a solution containing:

- oxygenated water: 10 µl of H_2O_2 in 110 volumes
- potassium acetate: 9.5 ml 0.05 M, pH 5.1
- 3-amino-9-ethylcarbazole: 2 mg dissolved in 0.5 ml of N-N'-dimethylformamide.

By this method we can dose nanogram per milliliter quantities of the aforesaid aminoglycoside.

The invention is obviously not limited to the methods of execution as described in the above

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examples and one can modify them without leaving the framework of following claims.

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CLAIMS

1 – Modified ATP resulting in covalent fixing on position 6, or preferably 8 of the adenine group of ATP, of a group M, chosen among those capable of a reaction of liaison with a chemical or immunological agent with a selective affinity for this group, by the intermediary of an arm of the type $-NH-(CH_2)_x-X$, or $-CO-(CH_2)_x-X$, where X is the group ensuring the liaison with the aforesaid group M and x varies from 2 to 20, mainly from 6 to 12 on the condition that x is different from 2 when M is a dinitro-2.4-phenyl group.

2 – Modified ATP as per claim 1, characterized by the aforesaid modification group, is capable of being specifically and directly recognized by another molecule or by a product itself easily detectable by a visualization method.

3 – Modified ATP as per claim 2, characterized by the aforesaid modification group, is constituted by an antigen or a hapten capable of being recognized already formed against this antigen or against this hapten.

4 – Modifed ATP as per claim 1, characterized by the aforesaid modification group used as a relay for another molecule or for another product capable of being visualized.

5 – Modified ATP as per any of the claims from 1 to 4, characterized by the aforesaid modification group or as per the case, the aforesaid relay molecule can be chemically coupled with a refined molecule marked by an enzyme and having a selective affinity for the said modification group or the said relay molecule.

6 – Modified ATP as per any of the claims from 1 to 5, characterized by the groups CH_2 of the

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aforesaid arm can in part be replaced by the CO or NH groups, if these replacement groups are not adjacent to the identical groups.

7 – Modified ATP as per any of the claims from 1 to 6 characterized by the aforesaid modification group, includes a group derived from biotin, avidin, or a group dinitro-2.4-phenyl.

8 – Detection method or dosage of a substance belonging to one category of biological substances such as certain antibiotics, mainly those belonging to the aminoglycocides group, which can, in the presence of specific enzymes, incorporate ADP or AMP, these molecules being capable of being supplied to these biological substances from ATP in the presence of an allotted specific enzyme, characterized by the steps as follows:

- to place the prepared compound with the researched substance with a reagent containing an ATP modified by a chemical molecule fixed in a covalent manner to the adenine cycle of this ATP and carrying at least one group not engaged in the aforesaid covalent liaison and capable of being coupled directly or indirectly with a molecule or a product with a specific affinity for this group and allowing it to be recognized, the said group also being one that does not prohibit the modified ATP that it carries to supply the modified AMP or modified ADP corresponding to a incorporable form in the said substance researched:
- to place, if need be, after elimination of the free modified ATP, the composition obtained as per the conditions, allowing the eventual coupling of modification groups carried by modified AMP or modified ADP incorporated with the said substance, with the molecule or the product, allowing the recognition of the modification group of the initial derivative of ATP, and

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- to eventually detect or to dose the said substance present carrying the said products of recognition.

9 – Procedure as per claim 8, characterized so that the reagent used contains modified ATP resulting in covalent fixing in position 6, or preferably 8, of the adenine group of ATP of group M, selected among those that are capable of having a reaction of liaison with a chemical or immunological agent with a selective affinity for this group by the intermediary of an arm of the type $-NH-(CH_2)_x-X$, or $-CO-(CH_2)_x-X$, where X is a group that ensures the liaison between a group M and x varies from 2 to 20, mainly from 6 to 12.

10 - Procedure as per claim 8, characterized so that the reagent used contains modified ATP as per any of the claims from 1 to 7.

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[refer to source for bilingual text – PDF pages 19 and 20]